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Research Article

Antibacterial Activity and Composition of Crude Extracts of Kaffir Lime (*Citrus hystrix* DC.) Leaves and Callus

Woro Anindito Sri Tunjung*, Desy Liana, Hennisa, and Lisna Hidayati

Laboratory of Biochemistry, Faculty of Biology, Universitas Gadjah Mada,
Jl. Teknika Selatan Sekip Utara Yogyakarta 55281 Indonesia

Abstract: Kaffir lime extracts contain bioactive compounds that have anti-bacterial properties. However, the production of bioactive compounds varies throughout the year, which limits its use as an antibacterial agent. Therefore, we have used callus induction, an in vitro culture technique, to provide controlled conditions for studying the activity of kaffir lime extracts from kaffir lime leaves and callus. Callus was induced from seed explants. Extraction of bioactive compound was performed by the maceration method using ethyl acetate or chloroform. Bacterial inhibition zone was determined using the Kirby-Baueur method with modifications. Analysis of bioactive compound was done using GC-MS. The results showed that all extracts had antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The most potent extracts against *S. aureus* and *E. coli* were generated using chloroform and ethyl acetate respectively. Furthermore, the antibacterial mechanism of kaffir lime leaves extracts was determined to be bacteriostatic. Both chloroform and ethyl acetate extracts had several bioactive compounds that inhibit bacterial growth. In addition, kaffir lime callus extracts inhibit *S. aureus* and *E. coli.* Together, these data reveal that kaffir lime callus also has broad spectrum antibacterial activity. Although the antibacterial activity of callus was less than that of leaves, these results warrant the further investigation of kaffir lime's callus to produce antibacterial agents.

Keywords: Anti-bacteria, callus, *Escherichia coli*, kaffir lime, leaves, *Staphylococcus aureus*

1. INTRODUCTION

The use of antibiotics to treat bacterial infection is associated with hypersensitivity, super-infection and organ toxicity [1]. Therefore, additional research is required to develop antibacterial agents that have fewer side effects on normal cells [2]. Natural products contain secondary metabolites which can be used as an antibacterial agent. Furthermore, secondary metabolites also perform an additional function of immunostimulant, so the consumption of a natural product can act directly to kill infectious agents and indirectly by increase the activity of immune system to eliminate those infectious agents [3].

Kaffir lime (*Citrus hystrix*), is a variety of citrus which is native to Indonesia, Malaysia and Thailand. Kaffir lime contains many secondary metabolites which can be used as an antibacterial agent such as flavonoids, alfa-tocopherol, limonoids, alkaloids, glycerophospholipids, terpenoids etc. [4, 5]. In general, production of secondary metabolites is affected by external factors such as biotic and abiotic environment [3]. Unfortunately, the content of bioactive compounds in natural medicines is unstable throughout the year which affects their use as a reliable method to treat bacterial infections [6]. Thus we need biotechnical approaches such as tissue culture to maintain the quality and even increase the quantity of secondary metabolites. The use of tissue culture for secondary metabolites production can be used as alternative method since we can control environmental factors [7].

This study examines the effects of kaffir lime extracts on both gram positive and gram negative bacteria. *Staphylococcus aureus* is gram positive bacteria which can cause dermatitis, acne pustule, nosocomial infection and toxic shock syndrome,

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* Corresponding Author: Woro Anindito Sri Tunjung; wanindito@ugm.ac.id

while *Escherichia coli* is gram negative bacteria which can cause diarrhoea, urinary tract infection and meningitis/sepsis. Natural products are categorized as having antibacterial activity if MIC (minimum inhibitory concentration) value < 1 mg mL^{-1} [8]. Therefore the objective of this study was to analyze the antibacterial activity of kaffir lime leaves and callus against *S. aureus* and *E. coli* and to determine the MIC value associated with their antibacterial properties.

2. MATERIALS AND METHODS

2.1 Preparation Simplicia Powder and Extraction

Samples of leaves were taken in Magelang, Central Java, Indonesia. The samples were then dried until a constant dry weight was obtained and made into a fine powdered kaffir lime leaves. Leaves powder was extracted by maceration method using methanol, ethyl acetate and chloroform to get paste extract. Then, the serial concentration of extract solution has been made by 100 % DMSO (dimethyl sulfoxide).

2.2 Induction and Cultivation of Kaffir Lime Callus

Callus induction was done according to induction method of callus Citrus rootstock with some modifications [9]. Seeds of kaffir lime were sterilised in 5.25 % Clorox for 3 min and rinsed twice with sterilised distilled water in Laminar Air Flow (LAF). Seeds were cut in half and induced on MS standard (Murashige and Skoog) medium which contain 8 g L^{-1} agar and 30 g L^{-1} sucrose. pH of the medium was adjusted in 6 by adding 1 N HCl and 1 N KOH. MS medium supplemented with growth regulator 2.4-D and BAP 1:0.5 mg L^{-1} was used to callus induction. Callus was taken after 40 days of incubation.

Callus was dried and then grounded into powder with mortar and pestle. Callus powder was extracted by maceration method. Firstly, we use methanol, ethyl acetate and chloroform extracts of leaves then empirical determinations were made to determine the best solvent for each bacteria. According to leaves experiment, callus was extracted using the best solvent for each bacteria. Ethyl acetate extract was used to test *E. coli* while chloroform extract was used for *S. aureus* experiment. The sample was put in solvent for 24 h. After that, sample solution was filtered through Whatman paper.

2.3 Medium and Samples Preparation

Antibacterial testing was conducted using Mueller Hinton Agar (MHA) which contained 0.2 % beef extract, 1.75 % acid hydrolysate of casein, 0.15 % starch and 1.7 % agar with final pH 7.3. Purification of bacteria was done to get 24 h bacterial culture. Then colony was taken and suspended in NaCl 0.85 % solution. McFarland 0.5 (1.5×10^8) was used for standardisation of some colonies [10].

2.4 Measurement of Inhibition Zone

Methanol, chloroform and ethyl acetate extracts were dissolved in DMSO 100 %. Well difussion method was used and was modified from Kirby-Baueur method according to NCCLS (*The National Committe for Clinical Laboratory Standard*) [10]. Tetracyclin 1 mg mL^{-1} was used as a reference. Clear area around extract was determined as inhibition zone. The best extract was determined based on the widest clear zone result among those extracts. Only the best extract was used for further experiments.

2.5 Growth Curve of Bacteria

The agar dilution method was employed to count the bacterial growth after extract exposure. Counting of the colony was made in 24 h, 48 h and 72 h after incubation. Then, the growth curve was made to determine whether the action mechanism characteristics of those extracts are bactericidal or bacteriostatic.

2.6 Analysis of Bioactive Compounds

Kaffir lime leaves and callus extracts were then analysed by GC-MS Shimadzu GCMS-QP 2010S, with a non-polar column AGILENT HP-5 MS with 95 % dimethylpolysiloxane and 5 % diphenyl polysiloxane to determine the profile of leaves bioactive compounds.

2.7 Data analysis

Data analysis was done using ANOVA ONE WAY

SPSS 16 and regression analysis.

3. RESULTS AND DISCUSSION

Kaffir lime (*Citrus hystrix*), is a variety of citrus which is native to Indonesia, Malaysia and Thailand. The taxonomical classification of *Citrus hystrix* DC. is

Kingdom	: Plantae
Division	: Spermatophyta
Sub Division	: Angiospermae
Class	: Dicotyledonae
Order	: Rutales
Family	: Rutaceae
Genus	: Citrus
Spesies	: *Citrus hystrix* DC [11]

3.1 Antibacterial Activity of Kaffir Lime Leaves

Inhibition zone of all of those extracts against *S. aureus* was shown in this Fig. 1 and Fig. 2. The result showed that all extracts have antibacterial activity against *S. aureus*. However, chloroform extract showed the best inhibition activities compared to other solvents.

Growth curve of *S. aureus* showed that inhibition mechanism of chloroform extract was bacteriostatic Fig. 3. Inhibition zone of all extracts against *E.coli* is shown in Fig. 4 and Fig. 5.

The result showed that all extracts have antibacterial activity against *E. coli* However,

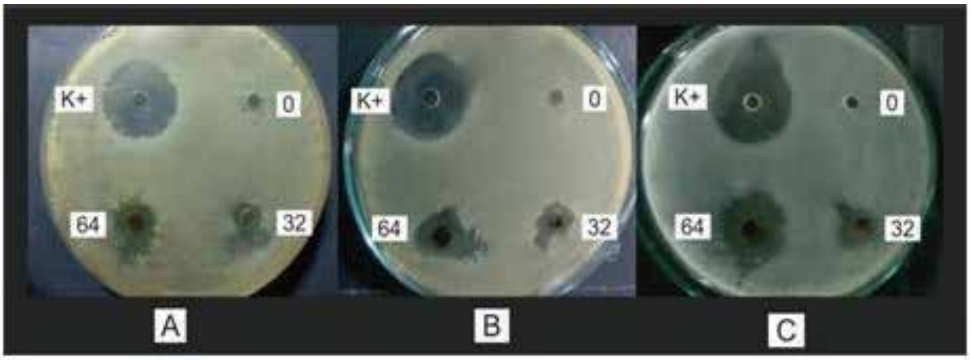

Fig. 1. Inhibition zone of methanol (A), ethyl acetate (B) and chloroform (C) leaves extract against *S. aureus* FNCC 0047

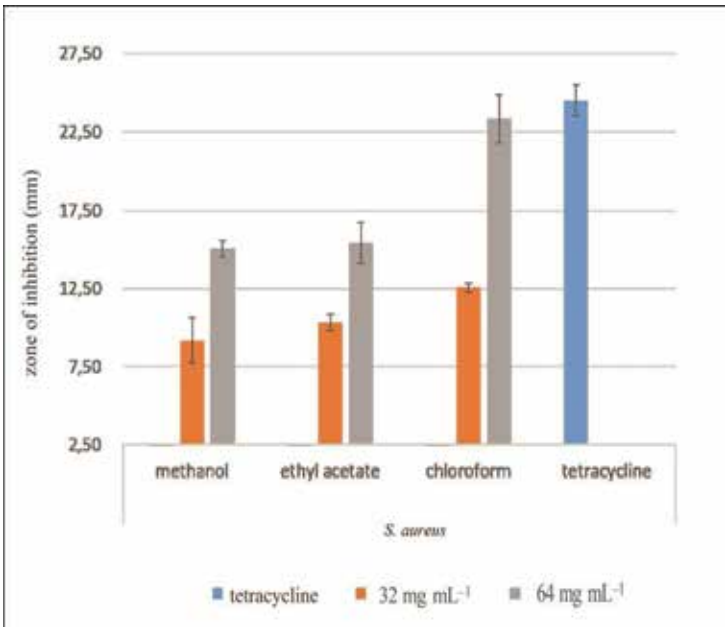

Fig. 2. Diameter of zone of inhibition of Kaffir Lime leaves extracts against *S. aureus* FNCC 0047

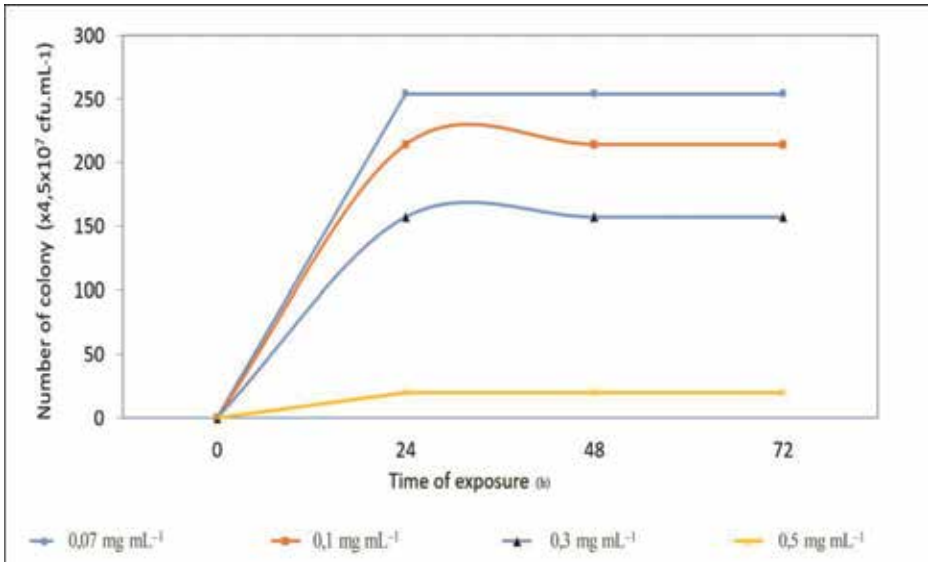

Fig. 3. Growth curve of *S. aureus* after treated with several concentrations of chloroform extract of kaffir lime leaves after 24 h, 48 h, and 72 h incubation

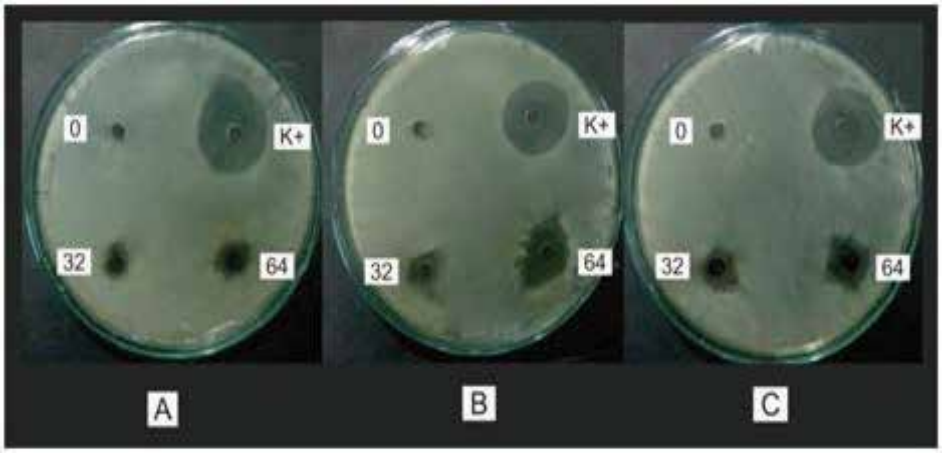

Fig. 4. Inhibition zone of methanol (A), ethyl acetate (B) and chloroform (C) leaves extract against *E. coli* FNCC 0091

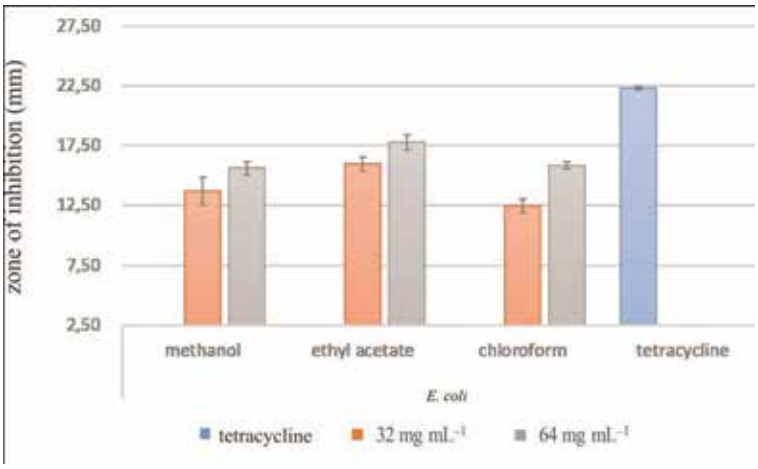

Fig. 5. Diameter of zone of inhibition of kaffir lime leaves extracts against *E. coli* FNCC 0047

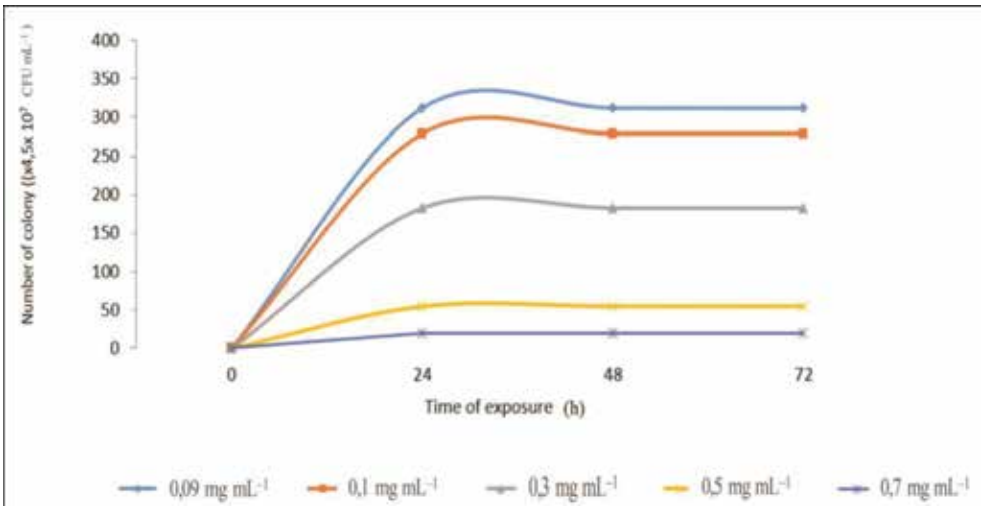

Fig. 6. Growth curve of *E. coli* after treated with several concentrations of ethyl acetate extract of kaffir lime leaves after 24 h, 48 h, 72 h of incubation

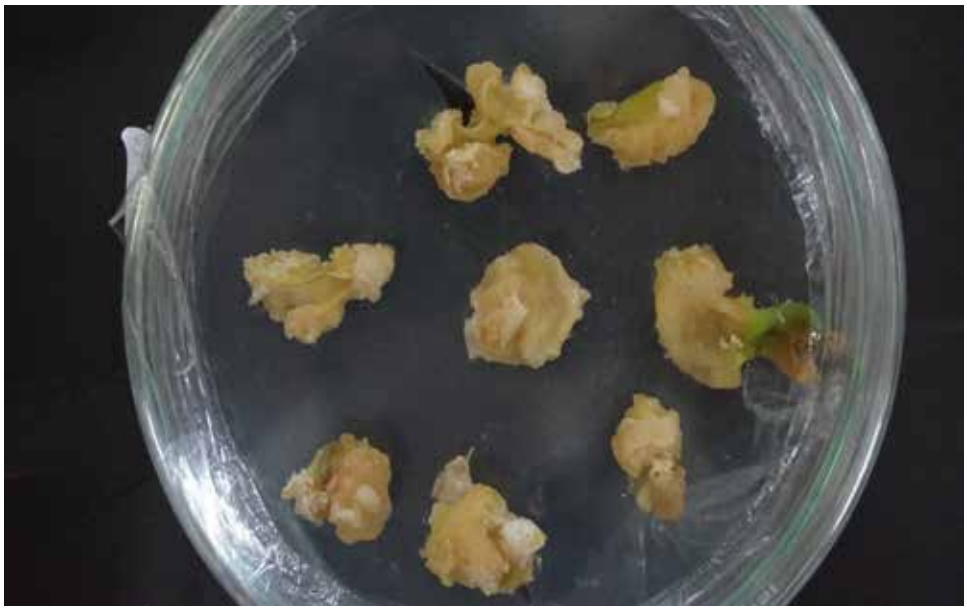

Fig. 7. Kaffir lime callus from seed explant

ethyl acetate extract had the best inhibition activity compared to other solvents.

Diameter of inhibition zone result can be used to categorize antibacterial activity. Natural products are categorised to be strongly active if the diameter zone is more than 18 mm, active if diameter zone is in the range of 13 mm to 18 mm, less active if diameter zone is in the range of 9 mm to 12 mm and inactive if diameter zone is less than 9 mm [8]. Ethyl acetate extract with concentration of 64 mg mL^{-1} produced inhibition zone (17.75 ± 0.66) mm against *E. coli*. Hence, ethyl acetate extract was categorised as active against *E. coli*. On the other hand, the same concentration of chloroform extract has produced inhibition zone (23.33 ± 1.52) mm against *S. aureus*. Therefore, chloroform extract had strong activity against *S. aureus*. This phenomenon showed inhibition in a dose-dependent manner, meaning that the activity of those extracts can be enhanced by increasing their concentration.

3.2 Antibacterial Activity of Kaffir Lime Callus

Kaffir lime callus can be produced by inducing seed in MS medium supplemented with growth regulator 2.4-D and BAP. The 2.4-D was synthetic auxin which can be used as herbicide and it can trigger cell to proliferate because of stress responses, while BAP is synthetic cytokine which has main function

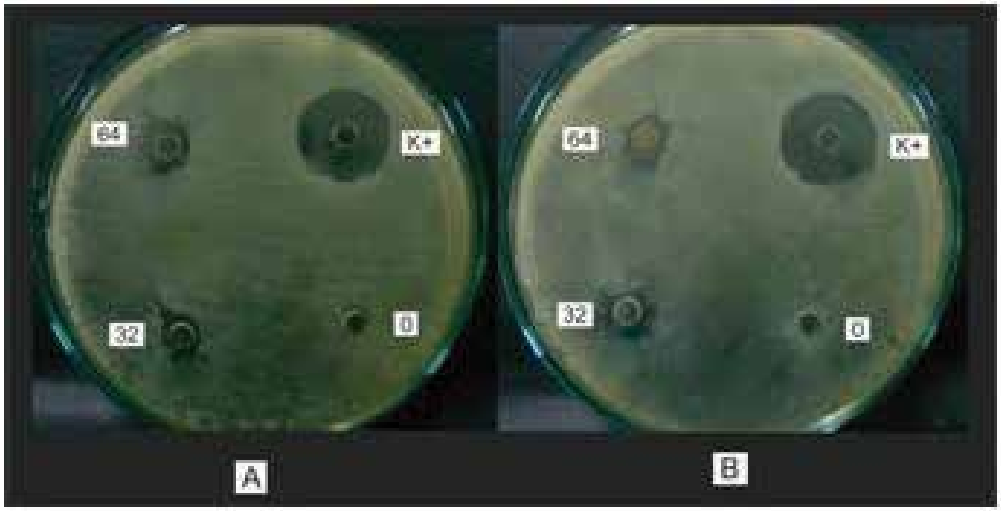

Fig. 8. Inhibition zone of kaffir lime callus ethyl acetate extract against *E. coli* and chloroform extract against *S. aureus* (concentration: 0 mg mL^{-1}, 32 mg mL^{-1}, 64 mg mL^{-1}) and tetracycline 1 mg mL^{-1} as reference.

Table 1. Inhibition zone of callus ethyl acetate extract against *E. coli*

Concentration (mg mL^{-1})	Zone of inhibition (mm)
64	13.75 ± 0.43^{b}
32	13.5 ± 1.25^{a}

* Note: Inhibition zone was including diameter of well (5 mm). Different letter showed significant difference

Table 2. Inhibition zone callus chloroform extract against *S. aureus.*

Concentration (mg mL^{-1})	Zone of inhibition (mm)
64	12.08 ± 0.94^{b}
32	11.58 ± 0.28^{a}

* Note: Inhibition zone was including diameter of well (5 mm). Different letter showed significant difference

in cell division.

Both callus extracts can be categorised as antibacterial activity. Based on the measurement of inhibition zone, the activity of callus extract can be categorised into several groups. At the concentration of 64 mg mL^{-1} (6.4 %), ethyl acetate extract of callus can be categorized to be active against *E. coli*, while the chloroform extract was less active against *S. aureus*. This phenomenon showed a dose-dependent relationship and suggested that the activity can be increased by adding more concentration of extract into bacterial culture.

The antibacterial activity of callus was less than than of leaves. Therefore it is important to do elicitation to callus to increase the content of secondary metabolite possessing antibacterial activity.

3.3 Bioactive Compounds of Kaffir Lime Leaves and Callus that Have Antibacterial Activity

Chloroform and ethyl acetate extracts of kaffir lime leaves were analyzed with GC-MS to determine bioactive compounds in kaffir lime leaves. In ethyl acetate extracts, there were found 52 bioactive compounds namely β-citronellol, phytol; citronellyl acetate; palmitic acid; linolenic acid; citronellyl propionate; nerolidol 2; α-farnesene; 1-methyl cyclohexene; citronellal; caryophyllene; delta-cadinene; hexacosane; eicosane; vitamin E; 1, 2, 3-propanetriol; monoacetate; 4-methyl-1,

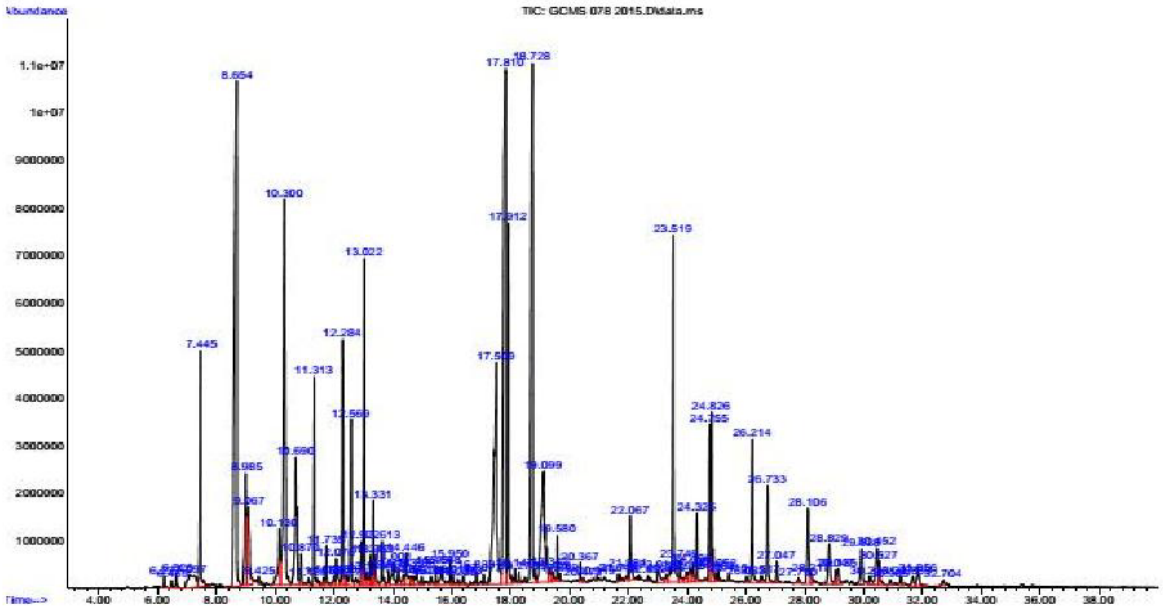

Fig. 9. GC-MS Chromatogram of ethyl acetate extract of *Cytrus hystrix* leaves

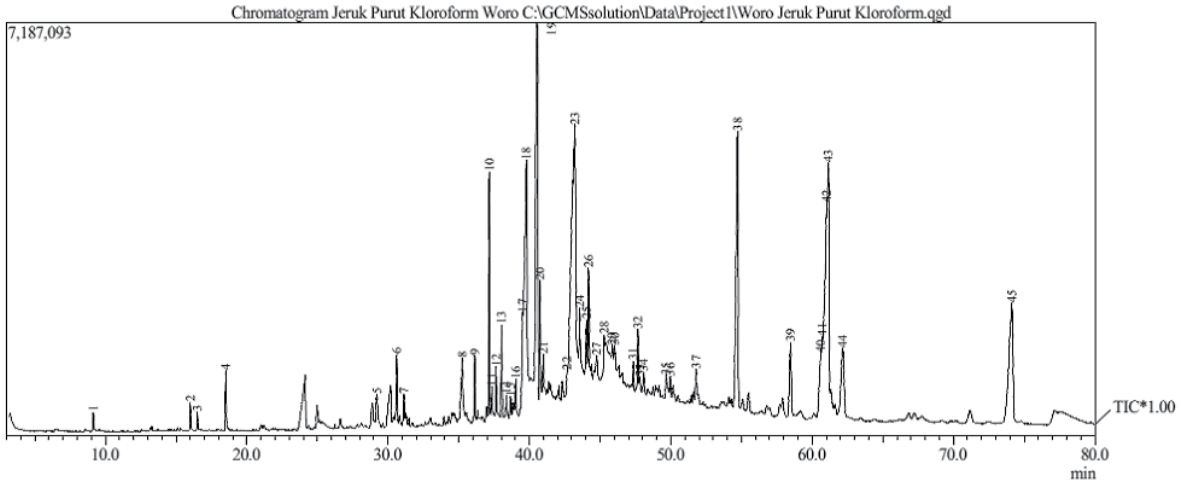

Fig. 10. GC-MS Chromatogram of chloroform extract of *Cytrus hystrix* leaves

4-heptadiene; γ-sitosterol; spathuleno; lupeol; squalene; torreyol/ cedreanol, germacrene; elemol; cetene; caryophyllene oxide; trans-farnesol; phytol acetate; hydroxyfuranocoumarin; α-caryophyllene/ α-humulene; ethyl palmitate; dotriacontane; dihydrolanosterol; neophytadiene; α-terpinolene, 1-octadecene (CAS) ; Z, Z-10, 12-hexadecadien-ol acetate; borane; dimethylmethyl (CAS), napthalene; citronellyl propanoate; 17-pentatriocontane; lupeyl acetate; margaric acid; trans-linalool oxide; dotriacontanol; 13-methyl-tetradec-13-ene-1,12-dio; farnesol; campesterol; benzenne propanoic acid; β-tochopherol and 1, 7-nonadiene, 4, 8, -dimethyl (Figure 9).

In chloroform extracts, 32 bioactive compounds were identified namely 9, 12, 15-octadecatrien-1-ol; 6-octen-1-ol, 3, 7-dimethyl; propionate; palmitic acid/ hexadecanoic acid; 1, 5, 9-decatriene, 2, 3, 5, 8-tetramethyl; heneicosane; neophytadiene; 14b-pregnane; heneicosane; neophytadiene; isophytol; 9-tricosene; citronellyl acetate; tetradecanoic acid/myristic acid; 2, 10-dodecadien-1-ol-3, 7, 11-trimethyl; 9-octadecanoic acid (CAS); phytol; cyclooctacosane; 3-eicosane; citronella; 6-octen-1-ol, phenol, 3, 5-bis(1,1-dimethylethyl); 2-hexadecen-1-ol, 3, 7, 11, 15-tetramethyl; 1-eicosanol; benzene,1-methoxy-2-[(4-methoxypheny) methyl] ; spathulenol;

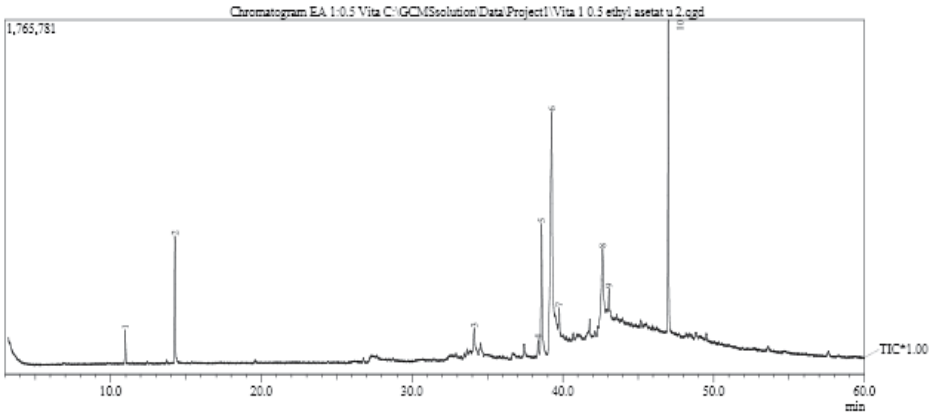

Fig. 11. GC-MS Chromatogram of ethyl acetate extract of *Cytrus hystrix* callus

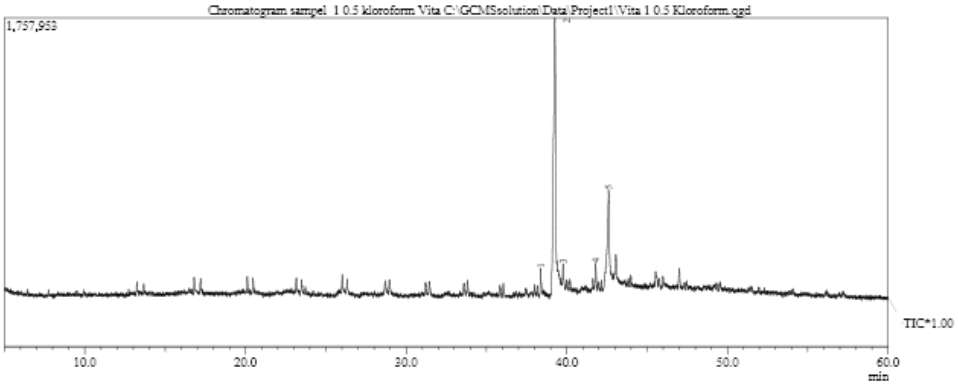

Fig. 12. GC-MS Chromatogram of chloroform extract of *Cytrus hystrix* callus

phytol isomer; trans-linaloloxide; citronellyl propionate; hexanedioic acid; 2-hexadecene, 3, 7, 11, 15-tetramethyl; 1, 2-benzenedicarboxylic acid, bis(2-ethylhexyl)ester; styrene; and eicosane (Figure 10).

The type of solvent in extraction can affect the yield, which will also make the determination of the amount of bioactive compounds in an extract difficult. Since bioactive compounds have different chemical characteristics and polarities they can or cannot be soluble in a particular solvent. Hence different solvent with different polarity can affect the efficacy of the extraction. According to the result, *S. aureus* was more sensitive by the exposure to chloroform extract compared to another extract, while the *E. coli* was more sensitive to ethyl acetate extract than another. This indicates that each extract contains a bioactive compound that is only preserved in a particular solvent.

Based on GC-MS analysis of chloroform extract, it was shown that those extracts contained secondary metabolite which was dominated with terpene, followed by fatty acids. According to the recent publication, it was known that chloroform leaves extract contains some potential antibacterial bioactive compounds, such as neophytadiene, squalene, isophytol, citronellyl acetate, myristic acid, 9-octadecanoic acid, phytol, 1-eicosanol, spathulenol, phytol isomer, trans-linalool oxide and eicosane. On the other hand, ethyl acetate extract contains some potential antibacterial agents such as beta-citronellol, phytol, citronellyl acetate, palmitic acid, linolenic acid, citronella, caryophyllene, eicosane, vitamin E, gamma sitosterol, spathulenol, squalane, germacrene, trans-farnesol, hydroxyfuranocoumarin, neophytadiene, alpha-terpinolene, naphthalene, trans-inalooloxide and farnesol. Although antibacterial mechanism for each compound remains unclear, previous studies showed that some secondary metabolites could damage bacterial membrane. This action belonged to farnesol [12].

On the other hand, ethyl acetate callus extract contained palmitic acid, oleic acid, alpha-pinene and 1.8-cineole (Fig. 11). However, any secondary metabolite was not detected in the chloroform extract (Fig. 12). In the chloroform extract hexadecanoic acid, palmitic acid and oleic acid had been detected. These fatty acids have antibacterial activity. These data supported previous data about inhibition zone between leaves and callus. The antibacterial activity of callus was less than that of leaves because secondary metabolite in callus was less than in leaves.

1.8-cineole is known to be anti-*E. coli* while alpha-pinene can act as antibacterial by damaging bacterial membrane. Alpha-pinene and 1.8-cineole belonged to terpenes groups with antibacterial property. The concentration of those compounds can be increased using some methods such as adding some elisitor to give a stress exposure or adding an enzyme precursor after making an analysis in their metabolic pathway [13]. Fatty acid such as palmitic was known to be able to inhibit the growth of bacteria by damaging cytoplasmic membrane [14].

4. CONCLUSION

Both leaves and callus extracts of kaffir lime had broad spectrum antibacterial activity which can inhibit the growth of *S. aureus* and *E. coli*. The best solvent against *S. aureus* is chloroform extract while for *E. coli is* ethyl acetate extract. Furthermore all tested extracts are bacteriostatic in their mechanism of action against each bacterial species. Although the antibacterial activity of callus is less than that of leaves, these results warrant further investigations of kaffir lime's callus to produce antibacterial agent.

5. ACKNOWLEDGEMENTS

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